DISSERTATIONES SCHOLA DOCTORALIS SCIENTIAE CIRCUMIECTALIS, ALIMENTARIAE, BIOLOGICAE. UNIVERSITATIS HELSINKIENSIS

IINES SALONEN

EXPLORING THE COMPOSITION, VARIATION AND INTERACTIONS OF UNICELLULAR ORGANISMS IN THE MARINE BENTHIC COMMUNITY WITH DNA METABARCODING

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Exploring the composition, variation and interactions of unicellular organisms in the marine benthic community with DNA metabarcoding

Iines Salonen

Doctoral dissertation

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Abstract

The marine benthic ecosystem, encompassing the sea floor and the sedimentary habitats within, plays a crucial role in major biogeochemical cycles and the functioning of the marine ecosystem. Despite its importance, many aspects of its ecology remain understudied, such as microbial community composition and bacteria–eukaryote interactions. In recent years, molecular ecology methods, in particular DNA metabarcoding, have provided us with new insights into benthic ecology. Compared with traditional methods, metabarcoding has the advantage of being applicable to various samples, generating large data sets and allowing reliable taxonomic identification without requiring morphological identification.

This thesis explores the use of the DNA metabarcoding method in examining the benthic ecosystem from different angles and targeting both eukaryote and prokaryote communities. 18S rDNA metabarcoding was used to target and track temporal variation in eukaryote communities in coastal sediment of the northern Gulf of Finland. The results demonstrate that DNA metabarcoding can be used to study sediment eukaryote community composition and variation over time. The key factor shaping the sediment eukaryote community was time, firstly the year and then the season, whereas location played a smaller role in explaining the community variation. In addition, the changes in the community composition could be linked to larger environmental phenomena, such as the timing and duration of the ice season, which in turn influenced the phytoplankton bloom. The ability of DNA metabarcoding surveys to resolve sediment community response to environmental factors indicates a potential for applications in biomonitoring and environmental assessment. Nevertheless, some limitations remain, such as the lack of standardization in metabarcoding methods and data analysis, and deficiencies in the reference databases.

In addition, 18S and 16S rDNA metabarcoding were employed to resolve trophic strategies and microbial interactions of a common benthic unicellular eukaryote, the foraminifera. Samples for these studies were collected from the intertidal mudflats of Texel Island in the Netherlands. Both intracellular bacteria and eukaryotes of foraminifera were targeted in these studies, as well as the foraminifera's own DNA, which allowed reliable genus-level identification. The intracellular eukaryote operational taxonomic units (OTUs) of different foraminiferal species reflected their trophic preferences: *Haynesina* sp. (genotype S16) and *Elphidium* sp. (genotype S5), which are likely to prefer an algal diet and/or are known to have a tendency for kleptoplasty, had an intracellular eukaryote content dominated by diatoms. In contrast, *Ammonia* sp. (genotype T6) contained also metazoan OTUs, implying potential predatory behaviour in addition to an algal diet. Based on these results, DNA metabarcoding can provide a comprehensive tool for the investigation of life strategies and ecology of even unicellular organisms, such as the foraminifera.

The intracellular bacterial OTUs of all foraminiferal species were enriched in sulphur-oxidizing and sulphate-reducing bacteria compared with the surrounding sediment bacterial community, where the relative abundancies of these bacteria were lower. The intracellular bacterial 16S OTUs of foraminifera were found to be species-specific, and the phylogenetic analysis of the sulphur-cycle related *aprA* OTUs showed that

some of these intracellular bacteria were closely related to known endobionts of other organisms. Therefore, the results suggest that intertidal benthic foraminifera may have a previously overlooked role in the benthic sulphur cycle.

Further research is needed to understand the exact role of the sulphur-cycle associated bacteria in foraminiferal ecology. For example, looking into the environmental conditions under which transcription of the sulphur-cycle genes takes place would enable assessment of their role and the potential foraminifera/endobiont contribution to the benthic sulphur cycle.

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I am deeply grateful to my supervisor, Dr. Karoliina Koho, for giving me the opportunity to join her research group. Karo introduced me to the fascinating world of foraminifera and sediment biogeochemistry. Together, we have had amazing adventures from the tidal mudflats of Texel to snowy winter sampling in the Gulf of Finland. Karo's support throughout these years has been pivotal to my work, and I want to thank her for giving me freedom and responsibility, which enabled me to grow and develop as a scientist. Warmest thanks also to my other supervisor, Dr. Elina Leskinen, for all the support and encouragement. I could have not wished for better supervisors to guide me in my scientific journey. I also wish to thank the members of my advisory committee, Prof. Kristina Lindström and Dr. Kirsten Jörgensen, for your valuable advice and support.

Our research group might have been small in size, but as a working environment, it has been creative, inspiring and productive. I am very grateful to Dr. Myrsini Chronopoulou for our long and fruitful discussions, as well as close collaboration over the years. I especially value Myrsini's vast knowledge on molecular ecology and her indispensable help with data analysis, but also her courage. Without Myrsini, we would have not been able to eliminate the cockroach that once terrorised our apartment in Yokosuka. I also wish to thank Dr. Jesse Harrison for his solid advice, calming presence and always being a valuable team member with excellent data analysis skills.

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I also want to thank all the people of the EcoEnv Research programme. Thank you Jaanika for your help and advice related to the DNA analysis. I want to thank Jukka for always having an open door and a willingness to help confused PhD students. Thanks also to the LUOVA doctoral programme, Anita Tienhaara for the help with the cover design of this thesis, and Cathryn Primrose-Mathisen for language revision. I also want to thank the technicians and everyone working at the Molecular Ecology and Systematics (MES) laboratory. Warm thanks also to the Institute of Biotechnology and Lars Paulin for all the help with DNA sequencing. In addition, I would like to thank the MERG community and lovely people from Tvärminne, especially Alf and Joanna. Special thanks also to Johanna for your support and friendship, and Eliecer for many great conversations.

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Table of Contents

List of original publications

This thesis is based on the following publications:

I Salonen, I., Chronopoulou, P.-M., Leskinen, E. and Koho, K. (2018). Metabarcoding successfully tracks temporal changes in eukaryotic communities in coastal sediments. FEMS Microbiology Ecology, 95(1).

II Chronopoulou, P.-M., Salonen, I., Bird, C., Reichart, G. and Koho, K. (2019). Metabarcoding Insights Into the Trophic Behavior and Identity of Intertidal Benthic Foraminifera. Frontiers in Microbiology, 10: 1169.

III Salonen, I., Chronopoulou, P.-M., Bird, C., Reichart, G. and Koho, K. (2019). Enrichment of intracellular sulphur cycle –associated bacteria in intertidal benthic foraminifera revealed by 16S and *aprA* gene analysis. Scientific Reports, 9(1).

The publications are referred to in the text by their roman numerals.

Author contributions

I The study was planned by KK, EL and IS. IS performed all the laboratory and data analysis. IS was responsible for interpreting the results with contributions from KK, EL and PMC. IS had prime responsibility for drafting the manuscript with contributions from PMC, KK and EL for the final version.

II KK conceived the study. KK and IS designed and carried out the sampling campaign and sample processing in the field, and GJR assisted with sampling coordination. IS was responsible for the isolation of living foraminifera. IS carried out the DNA extractions, as well as primer selection and testing. PMC was responsible for the final DNA amplifications and sequence data analysis. KK did the carbon and total nitrogen analysis. CB assisted with the protocol for foraminiferal DNA extractions and with phylogenetic analysis, and did the genotyping. IS and KK contributed to the data interpretation led by PMC. PMC drafted the manuscript with contributions from IS, KK, CB and GJR.

III KK conceived the study. KK and IS designed and carried out the sampling campaign and sample processing in the field, and GJR assisted with sampling coordination. IS was responsible for isolating living foraminifera and performing the majority of the laboratory and data analysis, except for the quantitative polymerase chain reaction and *aprA* analysis that were completed by PMC. KK completed the pore-water analyses. CB assisted with the DNA extraction protocol. IS was responsible for interpreting the results with the help of the other authors. IS was also responsible for drafting the manuscript with contributions from PMC, KK, CB and GJR for the final version.

IS = Iines Salonen, KK = Karoliina Koho, EL = Elina Leskinen, PMC = Panagiota-Myrsini Chronopoulou, GJR = Gert-Jan Reichart, CB = Clare Bird.

Abbreviations

aprA	dissimilatory APS reductase
bp	base pair
DNA	deoxyribonucleic acid
eDNA	environmental DNA
NGS	next-generation sequencing
OTU	operational taxonomic unit
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SOB	sulphur-oxidizing bacteria
SRB	sulphate-reducing bacteria
TEM	transmission electron microscopy

1. Introduction

1.1 Microbial ecology of the benthic ecosystem

Benthic ecosystems are some of the largest and most species-rich habitats on Earth (Snelgrove, 1999). Compared to the pelagic ecosystem, the benthic realm is characterized by limited horizontal mixing and pronounced physiochemical gradients based on oxygen availability and chemical zonation, which increase habitat complexity and heterogeneity (Pedersen et al., 2015). Higher number of microhabitats leads to niche diversification and resource partitioning, which in turn amplifies microbial diversity (Zinger et al. 2011). When temporal and spatial variability in physio-chemical factors increases, as for example when comparing coastal sediments to those of the nutrient-poor deep sea, increases also microbial diversity and variability (Zinger et al. 2011). Perhaps due to its complex nature, the benthic ecosystem is still among some of the least studied habitats, especially with regard to species diversity and ecosystem functioning (Snelgrove, 1999).

In the recent years, there has been a growing interest to investigate the microbial and unicellular life of the benthos, as these communities are known to play a crucial role in these ecosystems. For example, many global biogeochemical cycles, such as those of carbon, nitrogen and sulphur, are affected and mediated by processes taking place in the sediment that involve benthic microbial communities. These processes, where specific chemical reactions performed by sediment bacteria take place in a consecutive order in relation to sediment depth ultimately cause the chemical zonation of the sediment (Froelich et al., 1979). Although shedding light into benthic microbial communities would increase the understanding of the whole benthic ecosystem, there remains knowledge gaps concerning their diversity, variation and interactions.

In order to study benthic microbial communities and their role in sediment biogeochemistry and ecosystem functioning, reliable methods for their detection and identification are required. In the recent years, the development of molecular ecology methods such as next-generation sequencing (NGS) have been crucial in providing a more comprehensive view of the unicellular eukaryotic and prokaryotic marine benthic communities (Lozupone and Knight, 2007). One of its applications is the DNA metabarcoding, which combines NGS and DNA-based taxonomy, allowing the parallel sequencing and identification of several species coexisting in a bulk environmental sample, such as a sediment (Taberlet et al., 2012, Coissac et al., 2012). Therefore, it is able to generate large data sets rapidly (e.g. Coissac et al., 2012). The so-called barcodes are small hypervariable DNA regions of, for example, the ribosomal genes that are common for a broad range of species and provide enough resolution for a reliable identification (Coissac et al., 2012). At first, DNA barcoding efforts focused on assembling reference libraries of taxonomically well-known species (Ratnasingham and Hebert, 2007). This work was crucial in setting up curated reference databases, which now serve as the basis of reliable taxonomic identification of unknown species in metabarcoding surveys of environmental samples (Ratnasingham and Hebert, 2007). This efficient way of obtaining vast amounts of detailed data has enabled us to gain more comprehensive profiles on the benthic microbial communities, and little by little transformed our understanding of the benthic ecosystem diversity and structure. Overall, benthic microbial communities have been found to be very diverse both in species and function, and to still contain many unknown or poorly characterized

organisms (e.g. Forster et al., 2016, Lozupone and Knight, 2007).

DNA metabarcoding has many applications in marine benthic research (Fig. 1). The ability to resolve the community composition of a bulk environmental sample, like sediment, can be utilized to answer ecological questions related to species diversity, distribution and the genetic potential in the ecosystem (e.g. Bik et al., 2011, Massana et al., 2015, Forster et al., 2016, McGee et al., 2019). For example, targeting whole communities with metabarcoding methods and following their variation spatially or temporally, as well as targeting specific indicator species, can be applied to biomonitoring and environmental assessment. As metabarcoding does not require traditional morphological identification, it enables the detection of species that display cryptic morphologies or are in different life stages (Hebert et al., 2003, Coissac et al., 2012). Moreover, it may even be used to indicate the presence of unknown diversity and novel organisms (e.g. Corinaldesi et al., 2011, Forster et al., 2016, Sinniger et al., 2016). The accuracy and success of the identification depends on the comprehensiveness of the reference database, however their capacities are growing and improving constantly (Taberlet et al., 2012, Dell'Anno et al., 2015). The choice of the target region and the appropriate primers may be an arduous task as it may also influence the species captured as well as the taxonomic level obtained (e.g. Taberlet er al., 2012, Cahill et al., 2018, Ruppert et al., 2019). Taxonomic resolution may also vary depending on the species studied. Other limitations should also be kept in mind, including the fact that DNA metabarcoding is dependent on polymerase chain reaction (PCR) amplification steps, which can introduce biases (Pawluczyk et al., 2015). However, as DNA metabarcoding efforts continue to increase globally, so does our awareness of the potential pitfalls and the ability to improve study designs and reliability. In the future, there remains many possibilities for benthic metabarcoding studies including shedding light on prokaryote–eukaryote interactions, trophic strategies and food web structures, and investigating community compositions and biodiversity.

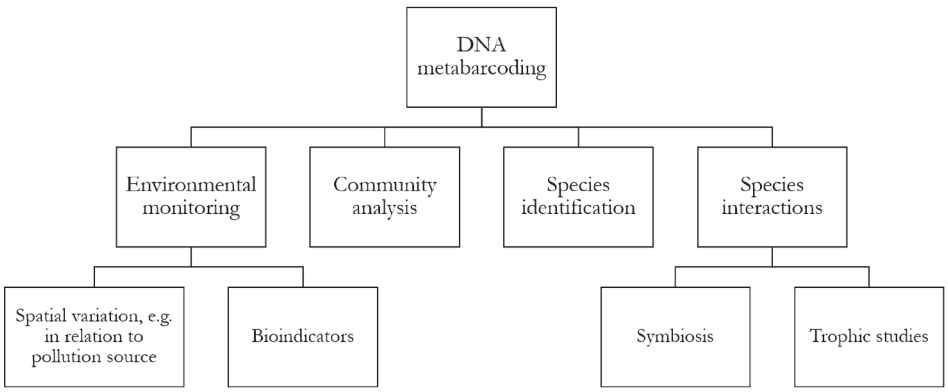

Figure 1. Potential applications of DNA metabarcoding in marine benthic research

1.2 Use of DNA metabarcoding in biomonitoring and environmental assessment

Many marine benthic ecosystems are under heavy anthropogenic pressures that threaten their ecological status. These pressures, such as global warming and eutrophication, cause environmental stress that can be manifested as regime shifts in these environments and ultimately the loss of biodiversity and ecosystem functioning (deYoung et al., 2008, Mora et al., 2013). In order to understand, protect and conserve these ecosystems, a number of national and international frameworks and initiatives exist. To estimate what measures should be taken to achieve and retain a good ecological status, sufficient monitoring and environmental assessment strategies play a key role (Pawlowski et al., 2018). Traditional monitoring of benthic marine environments relies on the morphological identification and calculation of macrofauna. This approach, however, is time-consuming, requires skilled taxonomic expertise, and is often limited to certain taxonomic groups. Meio- and microfauna, which are often excluded in the traditional approach, are typically more diverse and responsive to environmental changes than macrofauna (Kennedy and Jacoby, 1999, Brannock et al., 2016). The inclusion of smaller fauna in monitoring assessments would thus increase the resolution and diversity obtained. This approach may be particularly useful in environments low in macrofaunal diversity, such as the Baltic Sea (Bonsdorff 2006), deep-sea environments (Sinniger et al., 2016), or low-oxygen settings (Levin et al., 2009).

The application of DNA-based monitoring methods, such as metabarcoding, offers numerous practical advantages. Metabarcoding approaches are comprehensive, fast, relatively cost-efficient and easily applicable, and can perform as well in monitoring as traditional approaches (e.g. Lejzerowicz et al., 2015, Aylagas et al., 2018, Ruppert et al., 2019). The universal nature of this method could widen the scope of biomonitoring analysis, as in addition to macrofauna it is also able target meio- and microfauna. Furthermore, metabarcoding allows the detection of species without a priori knowledge, enabling the studying and monitoring of environments without assuming beforehand the species present, and even allowing the detection of new species (Corinaldesi et al., 2011, Valentini et al., 2016, Ruppert et al., 2019). As the sample size needed is smaller, it is also less destructive for the ecosystem (Valentini et al., 2016).

There exists a number of recent research efforts where the power of DNA metabarcoding in monitoring and environmental assessment has been demonstrated. For example, microbial eukaryotes have been shown to indicate a clear spatial community response to environmental disturbances and pollution sources, such as oil spills (Bik et al., 2012) and fish farming (e.g. Pawlowski et al., 2014, Lejzerowicz et al., 2015). In a similar way, sediment bacterial communities have also shown a clear change in diversity and community composition in relation to offshore oil and gas activities (Laroche et al., 2018). In addition to studying whole communities, metabarcoding approach can be used to target specific indicator organisms, such as specific groups of unicellular eukaryotes like foraminifera, diatoms, ciliates or testate amoebae (Pawlowski et al., 2016). For example, changes in foraminiferal diversity in response to fish farming or oil drilling have been demonstrated by Pawlowski et al., (2014) and Laroche et al. (2016) respectively, suggesting that these organisms are suitable indicators of environmental conditions and disturbances. Targeting bioindicators with metabarcoding can complement traditional methods (Apothéloz-Perret-Gentil

et al., 2017) or potentially even surpass them in efficiency (Pawlowski et al., 2014, Aylagas et al., 2018).

In addition to spatial variation, metabarcoding can be used to target temporal variation in benthic communities, allowing us to observe how communities change and react in response to environmental changes. These changes can for example be seasonal, such as the occurrence of the annual phytoplankton blooms, or larger regime shifts caused by anthropogenic stress. Temporal trends can for example be seen in coastal bacterial sediment communities in the Gulf of Finland that display seasonal variation in their composition, as some bacterial groups increase in relative abundance in response to organic matter inputs associated with the spring blooms or increased water inflow (Vetterli et al., 2015). Long-term surveys and monitoring studies are crucially important to detect potential regime shifts in species distribution and to distinguish potential anthropogenic impact from naturally occurring events. Temporal surveys can also be used to assess ecosystem recovery after environmental disturbances, such as oil spills (Brannock et al., 2017).

When conducting metabarcoding surveys on sediment, factors such as the age and origin of the DNA signal must be included in the study design (Goldberg et al., 2016). Compared to water column, DNA preserves well in sediments, especially when the conditions are anoxic (Coolen & Overmann 2007, Corinaldesi et al., 2011). Moreover, it has been estimated that also extracellular DNA can have a significant contribution to the amount of total DNA in sediments (Dell'Anno 2005). Thus, metabarcoding methods may be able to target signals of organisms even when they are not anymore present or viable in the sampling location (Goldberg et al., 2016). In the marine ecosystem, there is a constant flux of cells from the water column to the sediment, which contributes to the DNA signal retrieved from the sediment. For example, in diatom studies, sediment samples have been reported to be predominated by pelagic species, likely resulting from an abundance of recently settled dead cells from the water column, or resting stages (Piredda et al., 2018). Therefore, in addition to shedding light into benthic communities, benthic metabarcoding surveys can also be used to target events taking place in the water column, such as phytoplankton blooms, potentially increasing the value of this method in monitoring (Study I). Furthermore, recent studies suggest that targeting the ancient DNA in sediments with metabarcoding may be a useful tool in reconstructing past climatic and environmental events on long time scales, thereby corroborating traditional paleoceanographic methods (de Schepper et al., 2019).

1.3 Benthic foraminifera

Benthic foraminifera are single-celled eukaryotes that are common across marine habitats (Fig. 2). In some areas, they can contribute up to 50% or more of the benthic biomass (Snider et al., 1984, Moodley et al., 2000). Benthic foraminifera are well adapted to sediment heterogeneity and physiochemical gradients, and the vertical distribution of different foraminiferal species in the sediment typically reflects microhabitat preferences (Corliss, 1985). Foraminifera typically have a shell, also called a test, consisting of calcium carbonate or agglutinated material. The test's morphology can be used as a basis of identification, and they also preserve well in sediments creating extensive fossil records dating back to the Early Cambrian period (Culver 1991), and enabling the use of foraminifera in paleoceanographic

and paleoclimatic studies. In addition to analysing changes in the foraminiferal assemblage composition, which is a sensitive marker of environmental change, the geochemical signals preserved in the foraminiferal calcium carbonate provide another excellent paleoenvironmental archive. The test chemistry reflects the environmental conditions where the calcification took place, as well as the microhabitat preferences of the foraminifera (Schmiedl et al. 2004, Koho et al. 2017).

Due to their abundance and predominance, foraminifera are key players in the marine carbon cycle. They contribute by incorporating calcium carbonate in their shells, which eventually gets buried in the sediments along with dead individuals. Furthermore, as they also rapidly consume phytodetritus, they influence in that way how much carbon is buried in the sediment and removed from active cycling (Moodley et al., 2000, Woulds et al., 2007). Resource partitioning occurs among different foraminifera species coexisting in the same environment, especially in areas where foraminiferal biomass is high (Moodley et al., 2000). Different feeding strategies may include herbivory (Moodley et al., 2000, Nomaki et al., 2006), deposit feeding (Goldstein and Corliss, 1994), predation of other small eukaryotes (Dupuy et al., 2010) and even direct uptake of dissolved organic carbon (DeLaca et al., 1981). Bacteria can also be a food source for foraminifera (Goldstein and Corliss, 1994), but as such, they are not able to fulfil their carbon requirements (van Oevelen et al., 2006). In addition, foraminifera seem to take up bacteria only randomly, implying that bacterivory mainly occurs during deposit feeding (Nomaki et al., 2006). Some foraminifera appear to be more generalist feeders than others, feeding in an opportunistic manner on the easiest energy sources available, whereas others are more specialized, feeding on a particular food source (Wukovits et al., 2018). Certain foraminiferal species living in the photic zone can also perform kleptoplasty, which means that they harvest and maintain chloroplasts, using them for autotrophic energy acquisition, and thus expressing a mixotrophic feeding strategy (e.g. Bernhard and Bowser 1999, LeKieffre et al., 2018).

Key to the ecological success and wide distribution of benthic foraminifera is their ability to tolerate anoxic sediments and even withstand sulphidic conditions (Moodley et al., 1997, 1998, Langlet et al., 2013). This remarkable ability allows them to inhabit harsh, dynamic environments such as intertidal mudflats, seasonally hypoxic and anoxic settings, and to dwell deeper in the sediments. In anoxic conditions, foraminifera continue to grow and calcify and assimilate nitrogen and sulphur, implying that they are still active (Nardelli et al., 2014, Nomaki et al., 2016). One mechanism that allows benthic foraminifera to cope with anoxia is the ability of some species to collect and store nitrate intracellularly (Piña-Ochoa et al., 2010) and use it to perform complete denitrification in the absence of oxygen (Risgaard-Petersen et al., 2006, Woehle et al., 2018). However, not all foraminifera denitrify, even though they can survive in anoxic conditions for long time periods (Langlet et al., 2013). Instead, some of them may rely on dramatically reducing their metabolism and entering a state of dormancy, where they consume their intracellular energy reservoirs or even their own cytoplasm (LeKieffre et al., 2017, Koho et al., 2018). Alternatively, there may exist yet unknown foraminiferal metabolic strategies under anoxic conditions (Langlet et al., 2013). Such strategies may involve interactions with bacteria, as foraminifera are known to develop symbiotic connections with prokaryotes especially in oxygen-poor environments (Bernhard et al., 2018). These symbiotic prokaryotes have been suggested to include cyanobacteria (Bird et al., 2017), denitrifiers (Bernhard

et al., 2011) and sulphur-oxidizing bacteria (SOB) (Tsuchiya et al., 2015), among others. However, in most cases, the activity and metabolic function of these putative symbiotic bacteria are still unknown (Bernhard et al., 2018).

The application of molecular ecology approaches has considerable potential in offering new exciting insights to foraminiferal ecology. Recent pioneering research in this field includes studies resolving foraminiferal genetic diversity and genetic types (e.g. Darling et al., 2016, 2017), foraminiferal genomics (Glöckner et al., 2014, Woehle et al., 2018) and investigating microbiomes of photosynthetic larger benthic foraminifera (Prazeres, 2018, Martin et al., 2019). In the case of pelagic foraminifera, 16S rDNA metabarcoding has proven to be a useful tool in gaining insights into putative endobiotic relationships (Bird et al., 2017) as well as ecological strategies (Bird et al., 2018). A more mechanistic and complete understanding of foraminifera–bacteria interactions would help to resolve unanswered key questions of foraminiferal ecology related to their survival in anoxic conditions and their evolution. Comprehensive view on foraminiferal ecology would also shed light on the poorly understood role of single-celled eukaryotes in benthic biogeochemical cycles, as well as have implications for the use of foraminifera as paleoceanographic and paleoclimatic proxies.

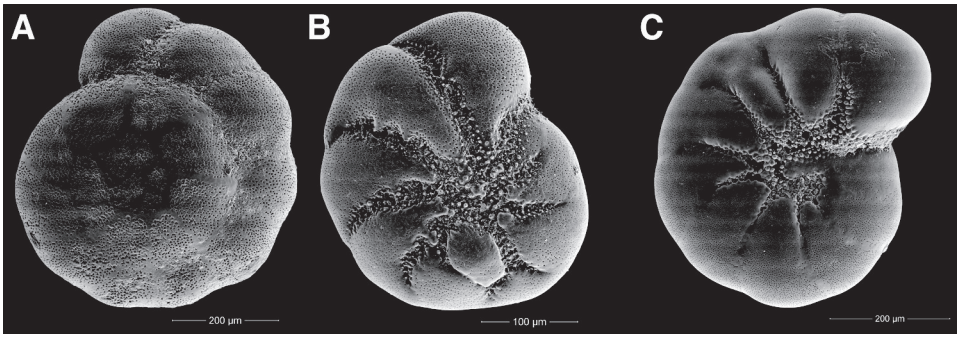

Figure 2. Scanning electron microscope (SEM) pictures of 3 benthic foraminifera species collected from intertidal mudflats of Texel Island, the Netherlands. A = *Ammonia* sp. (T6), B = *Elphidium* sp. (S5), C = *Haynesina* sp. (S16).

2. Aim of the thesis

The overall aim of this thesis was to apply molecular methods, in particular DNA metabarcoding, to gain insights into the ecology and functioning of the benthic ecosystem. As DNA metabarcoding is able to target even the smallest size fractions of the benthic organisms, which are often overlooked when using traditional microscopic methods, the focus of this research was on the unicellular organisms of the sediment rather than macrofaunal assemblages. This can have potential benefits, as including bacteria and unicellular eukaryotes in ecological studies can significantly widen our understanding of the ecosystem as a whole, as well as fill knowledge gaps in some key ecological questions.

This thesis consists of three studies, each focusing on a specific topic related to the application of metabarcoding in the study of the benthic ecosystem:

- Tracking temporal variation in sediment eukaryote communities and linking the variation to larger environmental phenomena in order to demonstrate the applicability of 18S rDNA metabarcoding in biomonitoring and environmental assessment.
- Resolving the identity and species-specific trophic interactions of intertidal benthic foraminifera using 18S rDNA metabarcoding.
- Examining the intracellular bacterial composition of intertidal benthic foraminifera and comparing it with the surrounding sediment bacterial community to detect species-specific patterns and potential bacteria–eukaryote interactions.

3. Materials and methods

3.1 Research areas and sediment sampling

3.1.1 Gulf of Finland (I)

In Study I, sediment samples from the northern Gulf of Finland, Baltic Sea, were collected in order to study the temporal variation of sediment eukaryote communities. Surface sediment samples were collected at two localities nearby Tvärminne Research Station, Storfjärden (33 m deep), and Muncken (11 m deep) (Fig. 3). Sampling was carried out seasonally (spring, summer, winter) during two consecutive years, 2008 and 2009. Samples were collected with a Gemax twin corer, and from each core (ø 9 cm) surface sediment was taken with a sterile plastic spatula and stored in –80°C in a deep freezer (see also Vetterli et al., 2015, for details).

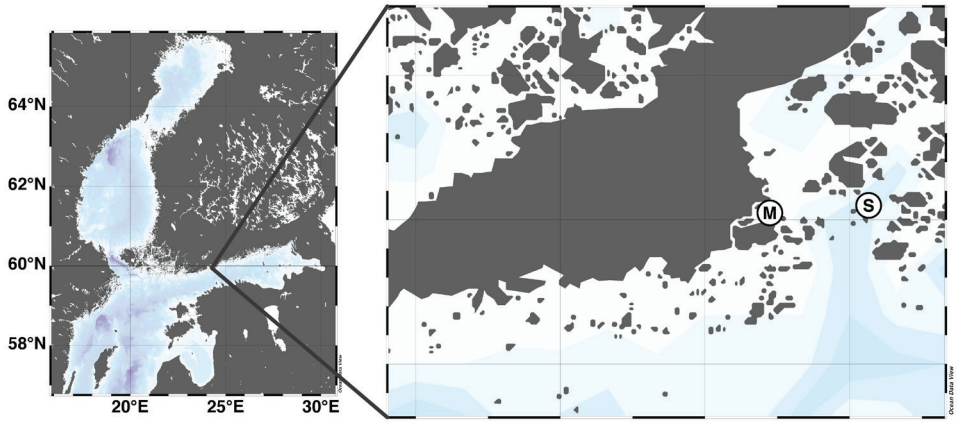

Figure 3. Map of the sampling sites in the Gulf of Finland. M = Muncken, S = Storfjärden. The map was created using Ocean Data View (Schlitzer 2020).

3.1.2 Texel, the Netherlands (II–III)

Sediment samples as well as foraminiferal specimens for Studies II and III were collected from two intertidal mudflat localities (Mokbaai and de Cocksdorp) on the island of Texel, the Netherlands (Fig. 4). Foraminiferal species *Elphidium* sp. (S5), *Haynesina* sp. (S16) and *Ammonia* sp. (T6) were sampled from site Mokbaai and *Elphidium* sp. (S5) from site de Cocksdorp. All these species are commonly encountered in intertidal mudflats (Moodley et al., 2000). Sediment cores from the two sites (ø 10 cm) were transported to the laboratory immediately after sampling and processed in an acclimatized room set at +12°C. First, oxygen and hydrogen sulphide were measured from the cores using Unisense microsensors (Unisense A/S, Aarhus, Denmark). Then, three sub-cores were taken from the cores with truncated syringes. Two of the sub cores were placed into an anaerobic glove bag, sliced at 1 cm intervals to 10 cm sediment depth, and from these slices pore water was extracted using centrifugation (III). The remaining sub core was also sliced at 1 cm intervals to 10 cm depth, and from each slice, a 1–1.5 g sample was taken with a sterile spatula for the analysis of the sediment bacteria/eukaryote community. The remaining sediment

slice was sieved, and foraminifera were picked under a stereomicroscope. Foraminifera were also preliminarily identified based on shell morphology. The picked specimens were washed in sterile artificial seawater to remove all external contamination and stored in RNAlater solution that dissolves calcite shell while keeping DNA intact until further processing.

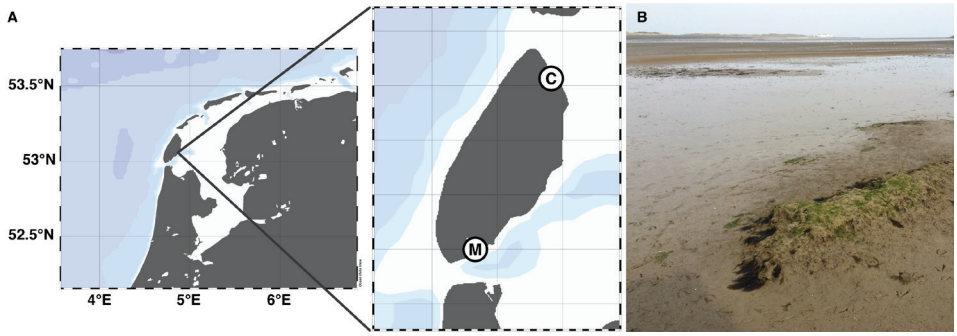

Figure 4. A = Sampling sites in Texel Island, the Netherlands. C is short for de Cocksdorp and M for Mokbaai. B = picture of the Mokbaai mudflat during low tide. Picture taken in May 2015.

3.2 DNA metabarcoding and qPCR (I–III)

3.2.1 DNA extraction

For both the sediment eukaryote (I–II) and bacterial community (III), the commercial PowerSoil® DNA Isolation Kit (MoBio, Carlsbad, CA, USA) was used, following the manufacturer's instructions. To extract DNA from single foraminiferal cells (II–III) the deoxycholate method was used (Holzmann and Pawlowski, 1996). Prior to DNA extractions, the foraminiferal naked cells, which were stored and decalcified in RNAlater, were carefully washed again to remove any shell remains or extracellular contamination (see Bird et al., 2017). In both sediment DNA and foraminiferal DNA extractions, negative control blank samples were processed to monitor and control possible contamination.

3.2.2 Amplification

After extraction of DNA, the desired hypervariable region was amplified with universal primers targeting either all eukaryotes or all bacteria (Table 1). To analyse eukaryote communities, universal primers targeting either the V4 or V9 hypervariable region of the 18S rRNA gene were selected (I–II). To study bacterial communities, universal primers targeting the V1–V3 region of the 16S rRNA gene were used (III). In addition, to capture sulphur oxidizers and sulphate reducers, the *aprA* (dissimilatory APS reductase) gene was amplified (III). In the amplification, negative controls were routinely analysed alongside samples. All amplifications were quality checked with agarose gel electrophoresis. Although DNA extraction blanks and negative controls did not produce a band on the agarose gel, they were nevertheless sequenced, and the resulting blank community was removed from the data set bioinformatically.

Table 1. Primers and target regions used in this thesis

Target	Gene, region	Study	Reference
Eukaryotes	18S rRNA, V4	I	Comeau et al., 2011 and Hugerth et al., 2014
Eukaryotes	18S rRNA, V9	I, II	Amaral-Zettler et al. 2009
Bacteria	16S rRNA, V1–V3	III	Salava et al., 2017
SOB and SRB	*aprA*	III	Meyer and Kuever, 2007

3.2.3 Sequencing and sequence analysis

Sequencing on the Illumina MiSeq platform was carried out at the Institute of Biotechnology in Helsinki, Finland. Prior to sequencing, samples were purified, amplified in a PCR reaction to add custom barcodes, which were later used to sort sequences into samples, repurified and pooled. The raw sequence reads were processed and trimmed using the Mothur software (version 1.36.1) following the standard operating procedure (Schloss et al., 2009) or in the case of *aprA* sequences, using the QIIME pipeline (version 1.9.1) (Caporaso et al., 2010). The bacterial and eukaryote sequences were aligned and identified against the SILVA reference database (release 132). For the foraminiferal identification (II), the PR2 database (version 4.7, Guillou et al., 2013) was also used together with the NCBI (National Center for Biotechnology Information) non-redundant nucleotide database to achieve genus-level identification. Eukaryote operational taxonomic units (OTUs) were determined using the 95% similarity threshold suggested by Caron et al. (2009). For bacterial OTUs, a threshold of 97% was used. Representative sequences of OTUs were determined in the Mothur software by choosing the sequence with the smallest distance to the other sequences of the clustering distance matrix. To avoid overestimating diversity, we plotted the cumulative sum of OTUs that would be filtered against the total counts and used them as threshold values for the final OTUs. Final OTU tables were created in R (version 3.4.2) using the package phyloseq (version 1.22.3). Statistical analysis and data visualization were also carried out in R using packages phyloseq (version 1.22.3), vegan (version 2.4-5) and ggplot (version 3.0.0).

3.2.4 Phylogenetic analysis (II–III)

For the foraminiferal identification, phylogenetic analysis based on representative sequences of the V9 hypervariable region of the 18S rRNA gene were used to verify and visualize the genetic placement of the foraminiferal specimens (II). In Study III, a phylogenetic tree of the *aprA* gene was constructed to compare the foraminiferal *aprA* OTUs with their closest relatives (> 85% similarity) and known free-living and symbiotic sulphur-cycle bacteria. In both cases, a maximum likelihood phylogenetic tree was constructed using MEGA7, after selecting the best substitution model according to the Bayesian information criterion. The tree was edited in Dendroscope (version 3.5.9) and Adobe Illustrator CC (version 23.0.2).

3.2.5 qPCR (III)

In order to investigate the metabolic functionality of the intracellular bacteria in foraminifera, quantitative polymerase chain reaction (qPCR) was used to quantify common genes related to the nitrogen and sulphur cycles. These genes were *amoA, nirS, nirK, norB* (nitrogen cycle) and *aprA, dsrB* (sulphur cycle). The same specimens were used for the qPCR analysis as for the 16S rDNA metabarcoding (Section 3.3.3). Triplicate reactions were performed for each specimen, and CFX Manager (version 4.0) software was used to determine absolute quantification of the targeted genes

4. Results and discussion

4.1 Temporal variation in sediment eukaryote communities and potential in biomonitoring (I)

18S rDNA metabarcoding was found to be a successful method in targeting and tracking temporal variation in sediment eukaryote communities. Based on our results, the factor driving the variation in the sediment eukaryote communities was time, mainly the year, followed by the season (Fig. 4 & 5, I). This contrasts with previous 18S rDNA metabarcoding studies, where spatial variation was the determining factor controlling for example meiofaunal communities in the Gulf of Mexico (Brannock et al., 2016). Here, the strong influence of the annual and seasonal changes on the sediment eukaryote community may be partially attributed to the fact that we captured not only benthic but also pelagic species, such as dinoflagellates and diatoms (Fig. 2, I). Sediment metabarcoding surveys can indeed provide an archive of events occurring in the overlying water column and offer insights into marine planktonic ecology and seasonality (Morard et al., 2017). The minor role of spatial variation in shaping the sediment eukaryote community may be linked to the fact that the majority of the eukaryote OTUs obtained in this study belonged to smaller size fractions, such as the single-celled diatoms and dinoflagellates (Fig. 2, I). The micro-eukaryotic fraction typically shows a more cosmopolitan and ubiquitous distribution than meiofauna or macrofauna (Bik et al., 2012, Fonseca et al., 2014). Currently, many existing benthic metabarcoding surveys targeting eukaryotes have focused on only targeting spatial variation, providing us only with a snapshot view of the sediment eukaryote community (e.g. Fonseca et al., 2014, Chariton et al., 2015, Laroche et al., 2018, Cordier et al., 2019). Temporal studies have been under-represented and the role of temporal variation in shaping the sediment community potentially underestimated (Brannock et al., 2016). Our study demonstrates that especially the annual changes can lead to dramatic shifts in the community composition. This leads to the conclusion that temporal variation should not be overlooked and that the importance of long-term surveys is crucial in drawing conclusions on the environmental status of a given location or detangling the anthropogenic impact from naturally occurring phenomena.

The temporal changes in our data sets could be linked to large-scale changes in predominant environmental/climatic conditions, such as temperature and length of the ice season, consolidating the potential of this method in long-term biomonitoring and environmental assessment. The inclusion of smaller sized fauna and pelagic as well as benthic species appears to increase biomonitoring capabilities, as the diversity obtained is higher, and it may increase the sensitivity of the study to target also temporal variation. The potential of metabarcoding in biomonitoring has also been established in a number of studies (reviewed in Ruppert et al., 2019), some of them even using comparative approaches to show that metabarcoding can perform as well as traditional monitoring methods (Aylagas et al., 2018). Monitoring aims always depend on the environment, and therefore monitoring programmes are often best designed regionally or in relation to a specific target. However, based on the research presented here, some general recommendations on the use of metabarcoding to target and monitor sediment eukaryotes can be made. Firstly, to gain a comprehensive view of the sediment ecosystem and to target bigger, overarching environmental trends, long-term surveys are advisable. Secondly, the careful choice of sampling period is

required to capture the relevant signal, as seasonality also plays a role in shaping the community.

For metabarcoding to perform reliably and reproducibly in biomonitoring surveys, there are some concerns from sampling to DNA extraction, amplification and bioinformatical analysis which should be taken into account (reviewed in e.g. McGee et al., 2019, Zinger et al., 2019). To obtain the whole community structure, for example, in marine sediments, a sufficient sampling size is crucial. Many previous studies, including ours here, have used a small amount of sediment as sample material (typically for one kit extraction, 0.25 g of sediment is needed). However, recent studies show that the sediment volume should be as high as 14 g to reach a satisfying level of diversity, especially when targeting larger eukaryotes such as metazoans (Nascimento et al., 2018). Based on our data and the similarity of the replicates, even smaller sample volumes may be sufficient to capture the unicellular eukaryote diversity and the temporal changes they indicate, since these organisms are likely to be more homogenously distributed in a smaller volume of sediment. In addition to the sample volume, DNA extraction is an important step, which can impact the diversity, and in which a sufficient number of negative controls should be used in order to monitor possible contamination (Zinger et al., 2019).

One of the key questions in metabarcoding surveys is the choice of the targeted DNA region and primer design, which can significantly influence the resolution and coverage of the survey (Ruppert et al., 2019). Selecting the target region is a balancing act between obtaining a good resolution and still capturing as many taxonomic groups as possible. For example, the cytochrome oxidase I gene may offer a greater taxonomic resolution than the 18S rRNA gene (e.g. Wangensteen et al., 2018), but it may not be able to capture as many phyla and fail to target certain groups, such as molluscs, efficiently (Cahill et al., 2018). The reason why the 18S rRNA gene is often reported to provide more taxonomically assigned OTUs may be attributed to the fact that more 18S rRNA sequences can be found in reference databases (Cordier et al., 2019). This situation, however, is likely to improve in the future, as sequencing efforts keep increasing.

The 18S rRNA gene has several variable regions, which also provide slightly different results. Here, we observed that although regions V4 and V9 were able to track the same overall trends in community structure, including seasonal and annual changes, the V9 region of the 18S rRNA gene was able to better target and resolve one dominant eukaryote group, class Maxillopoda (Fig. 2, I). Despite being shorter than the V4 region, V9 has the advantage of capturing almost all eukaryote phyla (Pawlowski et al., 2011). Thus, for wider community metabarcoding analysis, a universal target region such as the 18S V9 is recommended, even though the final choice always depends on the scope of the study. In addition, increasing sequencing depth and the number of replicates improves the coverage and diversity obtained (Lanzén et al., 2017). An optimal number of replicates is hard to establish, as it depends on the study environment and the study aim (Lanzén et al., 2017). In the end, as Ruppert et al. (2019) pointed out, due to the nature of eDNA, which consists of tiny fragments unevenly distributed in the environment, streamlining DNA metabarcoding methods will always be more about minimizing and understanding potential errors than achieving perfect detection.

4.2 Intracellular eukaryote composition of foraminifera – trophic interactions (II)

Despite sharing the same habitat, intertidal benthic foraminifera have species-specific intracellular eukaryote content, as verified here by the 18S rDNA metabarcoding approach (Fig. 2, II). Furthermore, the eukaryote content of foraminifera did not closely reflect that of the surrounding sediment, and the alpha diversity of the sediment communities was significantly higher compared with the intracellular foraminiferal communities (Fig. 3, II). This evidence rules out the possibility of foraminifera only relying on random feeding of the surrounding sediments and ambient eukaryotes, as then the intracellular eukaryote OTUs would be expected to more closely mimic that of the surrounding sediment community. Whereas the sediment eukaryote community was affected by the sampling site, the main driving factor of the foraminiferal intracellular eukaryote OTUs was the species, regardless of the site (Fig. 4 & 5, II).

Haynesina sp. (S16) and *Elphidium* sp. (S5) had an intracellular eukaryote content consisting mainly of diatoms, implying a preference for algal diet (Fig. 2, II). This confirms previous research (e.g. Moodley et al., 2000, Nomaki et al., 2006, Schönfeld and Numberger, 2007) stating that these species are mainly planktivorous. Furthermore, this result may be linked to kleptoplasty, allowing the host foraminifera to perform photosynthesis, which can occur in both of these species (Jauffrais et al., 2018, Pillet et al., 2011). Experimental studies with *Haynesina germanica* have indicated that this species may prefer mixotrophy to algal diet (Wukovits et al., 2018). 18S rDNA metabarcoding verified that the intracellular diatom OTUs of *Haynesina* sp. (S16) and *Elphidium* sp. (S5) came from various sources, suggesting that there is no strong selectivity over the algal food/kleptoplast source. These foraminifera may even keep a pool of different kleptoplasts, allowing the foraminifera to choose and shuffle the most appropriate kleptoplast under environmental stress, a strategy previously suggested for the photosymbiont-bearing foraminifera *Pararotalia calcariformata* (Schmidt et al., 2018).

In feeding experiments, intertidal *Ammonia tepida* has expressed a generalist behaviour and a preference for soft chlorophyte food sources over harder diatom detritus (Wukovits et al., 2018). The results of our study confirm that *Ammonia* sp. (T6) seems to be primarily a secondary consumer of the benthic ecosystem, preferring both algae and other small eukaryotes in its diet (Fig. 2, II). However, the intracellular enrichment of metazoan classes in our specimens implies that *Ammonia* sp. (T6) also displays predatory behaviour towards small eukaryote classes, such as nematodes (Fig. 2, II). Predation of nematodes has been previously observed among benthic foraminiferal species *Globobulimina auriculata* and *Globobulimina turgida* (Glock et al., 2019). In the case of *Ammonia* sp. (T6), predatory behaviour towards nematodes has also been documented previously but only in laboratory experiments (Dupuy et al., 2010). Our results confirm that predation of metazoans may be a common trophic strategy for foraminifera in their natural habitat as well, at least for *Ammonia* sp. (T6).

Many previous studies investigating foraminiferal trophic strategies have been based on isotope labelling approaches and laboratory feeding experiments (e.g. Moodley et al., 2000, van Oevelen et al., 2006, Nomaki et al., 2006, Wukovits et al., 2018). These studies have helped to elucidate the complex and diverse feeding strategies of benthic foraminifera (see introduction Section 1.3). However, a non-experimental

method such as DNA metabarcoding is needed to provide us with *in situ* data that can verify the trophic behaviour that occurs in the natural habitats of different foraminiferal species. As a method, it offers numerous advantages, such as providing high taxonomic resolution and reliable identification of various food sources. In addition, as metabarcoding does not require a priori knowledge of the investigated organisms, it is able to detect previously overlooked trophic preferences. Furthermore, it can be applied to single individuals, which permits taking intraspecific variation into account.

4.3 Metabarcoding-based identification of foraminifera (II)

Traditionally, foraminiferal identification has been based on the morphology of their tests, typically consisting of calcium carbonate or agglutinated material. However, this method has its pitfalls, for example, it requires a high level of taxonomic expertise. Furthermore, characteristics of the foraminiferal tests are often quite limited, and they can display a significant amount of variation (Pawlowski and Holzmann, 2008). Especially small individuals may be difficult to identify based on morphology. Foraminiferal species can also have many different genotypes that may inhabit the same areas and that are difficult to distinguish morphologically (e.g. Schweizer et al., 2011, Pawlowski and Holzmann, 2008). Finally, not all foraminifera have hard shells, as some species (namely soft-shelled monothalamous foraminifera) have an outer surface consisting only of a relatively thin organic wall with very little morphological features.

Foraminifera have very divergent and insertion-rich ribosomal genes, making their identification with regular universal primers sometimes challenging (Pawlowski, 2000, Pawlowski and Lecroq, 2010). Nevertheless, DNA metabarcoding has proven to be a very promising tool in foraminiferal identification, and it has revealed that foraminiferal diversity is greater than previously recognized and that many undescribed species may still exit (Pawlowski and Holzmann, 2008). The foraminifera-specific 37f region is commonly used to achieve species-level identification, as despite being short, it provides reliable and accurate resolution and identification (Pawlowski and Lecroq, 2010, Pawlowski and Holzmann, 2014). However, this region is still under-represented in public reference databases, making other regions, such as the V9 hypervariable region of the 18S rRNA gene used here, a compelling option. The V9 hypervariable region of the 18S rRNA gene is relatively short (only approximately 130 bp in length), but it is able to target almost all eukaryotic phyla and amplify even the foraminiferal ribosomal genes that are very variable and complex (Pawlowski and Holzmann, 2008; Pawlowski and Lecroq, 2010; Pawlowski et al., 2011). Based on our results, the V9 region of the 18S rRNA gene can provide sufficient resolution for foraminiferal identification (Table 1, II). This method can distinguish foraminiferal species to genus level, and when combined with phylogenetic analysis, it can be used to infer/visualize the genotypes of the foraminifera (Fig. 1, II). Furthermore, metabarcoding surveys of foraminifera, targeting the 18S V9 region as a basis for foraminiferal identification, have the advantage of also targeting other eukaryotes, allowing simultaneous analysis of trophic strategies.

4.4 Intracellular bacterial composition of intertidal benthic foraminifera (III)

16S rDNA metabarcoding successfully targeted the intracellular bacterial composition of intertidal foraminifera, which was found to be species-specific (Fig. 2 & 5, III). Sediment bacterial community was influenced by the site and depth, whereas the intracellular bacteria in foraminifera were dictated by the foraminiferal species (Fig. 5 & 6, III). Although similar bacteria were present in the sediment and in the foraminifera, they showed contrasting relative abundances (Fig. 2, III). If bacterial enrichment in foraminifera was completely random and linked to deposit feeding or generalist feeding patterns, we would not expect to see clear enrichment of certain bacterial taxa over others but a closer resemblance of the intracellular bacterial OTUs to the surrounding sediment bacterial community. Here, a link to the sediment community exists, but species remains the determining factor of the intracellular bacterial OTUs of foraminifera regardless of site and depth, which in turn affect the surrounding sediment bacterial community. Moreover, previous research has indicated that these particular foraminiferal species likely prefer herbivory, mixotrophy and predation as a trophic strategy, instead of bacterivory or detrivory (Wukovits et al., 2018, Chronopoulou et al., 2019).

Compared with the surrounding sediment bacterial community, sulphur-cycle bacteria, sulphate-reducing bacteria (SRB) and SOB were clearly enriched within the foraminifera (Fig. 2, III). In contrast, nitrogen-cycle bacteria played a trivial role in our specimens, as they were neither a relatively abundant part of the intracellular bacterial OTUs, nor were the common nitrogen-cycle genes detected with qPCR. The analysis of the intracellular *aprA* OTUs revealed that the intracellular sulphur-cycle bacteria were species-specific. The intact nature of a relatively long DNA fragment obtained suggests that the intracellular bacterial DNA was not all fully digested, that is to say, food related (Pompanon et al., 2011). In addition, the presence of intact and dividing intracellular bacteria has been previously observed at least in *Ammonia* sp. (T6) (Koho et al., 2018).

Currently, the role of foraminifera in the sulphur cycle is understudied. Sulphate-reducing intracellular bacteria have been previously recorded in the benthic foraminiferal species *Virgulinella fragilis*, implying that they could be endobiotic (Tsuchiya et al., 2015). Additionally, *Ammonia* sp. has been shown to incorporate elementary sulphur under dysoxia, potentially utilizing it to synthetize sulpholipids through a sulphate activation pathway, which could potentially benefit intracellular sulphur-cycle bacteria (Nomaki et al., 2016). Phylogenetic analysis of the *aprA* gene showed that many of the intracellular foraminiferal *aprA* OTUs were closely related to symbiotic sulphur-cycle bacteria of other marine eukaryotes, such as ciliates, thus implying a putative endobiotic interaction (Fig. 7, III).

Sulphur-cycle related symbionts are commonly found in anoxic, sulphidic marine environments, in association with eukaryotes such as ciliates (e.g. Edgcomb et al., 2011), nematodes (Polz et al., 1992) and shrimps (Ponsard et al., 2013). SOB symbionts are usually suggested to fix carbon autotrophically while oxidizing inorganic sulphur compounds into sulphate and thiosulphate (Stewart et al., 2005, Dubilier et al., 2008), although some of them may also be heterotrophic and take up organic carbon (Ponsard et al., 2013, Seah et al., 2019). SRB symbionts, in turn, produce sulphide by oxidizing organic or inorganic compounds. Endobiotic connections

between eukaryotes and SRB or SOB often occur in challenging environments such as the deep sea or intertidal mudflats (Ruehland and Dubilier, 2010). In ciliates, for example, endobionts have the ecological role of helping the host to adapt and survive in periodically anoxic benthic environments by increasing energy yields and recycling resources in carbon-limited conditions (Edgcomb et al., 2011, Seah et al., 2019). In a similar way, foraminifera could benefit from sulphur-cycle symbionts in the intertidal mudflats. SOB symbionts can also provide additional benefit to the host through a so-called 'nutritional symbiosis', as eventually they may get eaten by the host (Stewart et al., 2005). In foraminifera, the function of these putative sulphur-cycle endobionts is still unclear, but based on previous studies on sulphur-cycle symbioses in other eukaryotes, we hypothesize that their metabolic role could be related to the acquisition of organic carbon and other key compounds, such as amino acids. To resolve the activity, function and distribution of these intracellular bacteria, and ultimately to verify an endosymbiotic relationship and elucidate its meaning for the host's survival, further analyses are required. Such analysis can include, for example, the analysis of RNA transcriptomes to indicate the activity of putative endobiotic bacteria and to gain insights into their functions or TEM imaging. In addition, methods like fluorescence *in situ* hybridization (Amann et al., 1990) are recommended to gain insights into the activity and distribution of potential endobionts. Ultimately, to acquire a comprehensive view of the host–endobiont complex and its evolutionary origin, genomic studies are advisable (Nowack and Melkonian, 2010, Worden et al., 2015).

Symbioses between unicellular eukaryotes and bacteria likely have evolutionary significance for the host by providing them new biochemical functions (Nowack and Melkonian, 2010). Endobiotic connections related to sulphur-cycle have developed several times in the evolutionary history of eukaryotes and are estimated to be more diverse and abundant than what is currently understood (Bernhard et al., 2000, Dubilier et al., 2008). Foraminifera are evolutionary old eukaryotes that display a variety of life strategies and are masters of survival under challenging environments. As explained in the introduction (Section 1.3), there are still many unanswered questions related to foraminiferal life strategy and survival in anoxic conditions. Much of the knowledge of foraminifera–bacteria interactions is based on transmission electron microscopy observations instead of molecular evidence, and therefore the metabolic function of the putative endobiosis remains unclear (Bernhard et al., 2018). Furthermore, unlike in other marine unicellular eukaryotes, the genomics of foraminifera are still largely unresolved, which is why we are possibly only beginning to understand the evolution, metabolic abilities and endobiotic interactions of these fascinating organisms. So far, only one foraminiferal genome belonging to the freshwater species *Reticulomyxa filosa* has been annotated, and it has shown evidence of lateral gene transfer between foraminifera and bacteria, implying a long history of co-evolution and interactions (Glöckner et al., 2014). The evidence presented here, indicating the strong presence of a diverse intracellular SOB and SRB in intertidal foraminifera, supports the idea that foraminifera could have potential endobiotic connections with sulphur-cycle bacteria. Potentially, these communities might provide the foraminiferal host additional biochemical pathways and enable it to colonize challenging environments limited by oxygen but rich in sulphate and sulphide.

5. Conclusions and future perspectives

The studies presented in this thesis demonstrate, how the application of metabarcoding methods can in many ways shed light into benthic ecosystem diversity, functioning and interactions. It has been estimated that the benthic ecosystem still contains many unknown and understudied species, especially when it comes to microbial/unicellular life (Forster et al., 2016). Metabarcoding offers an efficient way to gain large amounts of detailed information, and capture signals that may have been previously overlooked, opening new research horizons. In addition to resolving the benthic species diversity, metbarcoding approaches can offer insights into the ecology, evolution and interactions of specific species.

Currently, research on feeding strategies and trophic behaviour increasingly utilizes DNA metabarcoding applications (Alberdi et al., 2019). This method has already been used to distinguish and determine resource partitioning and feeding strategies of larger eukaryotes, such as coral reef fish (Leray et al., 2013), lizards (Pereira et al., 2019) and large African herbivores (Kartzinel et al., 2015). Here, we provide evidence that 18S rDNA metabarcoding is a suitable method for resolving trophic strategies of much smaller organisms, such as the single-celled foraminifera. Different feeding patterns between species result from different tropic/survival strategies, and these differences must be taken into account when assessing the effect foraminifera have on benthic carbon and nitrogen fluxes and organic matter turnover rates (Wukovits et al., 2018). Combining DNA metabarcoding with other analyses, such as TEM imaging or feeding experiments, has enormous potential in providing accurate and reliable descriptions of feeding behaviour of different species. In the case of the foraminifera, studying their ecology is particularly important, as they are among the most widely applied proxies used in paleoceanographic and paleoclimatic research. Their habitat and lifestyle has direct influence on geochemistry of their shell, which is used in reconstructions of past climatic conditions (Schmiedl et al., 2004, Koho et al., 2017) Thus, a more mechanistic and comprehensive understanding of their ecology and evolution is required, to secure the correct interpretation of the paleoenvironmental data.

The mechanisms of coexistence and species interactions are key questions in understanding benthic ecosystem functioning and resilience. The results of this thesis suggest that DNA metabarcoding can be used to directly investigate the *in situ* interactions between benthic microbial communities. For example, as shown in Study III, this method can provide a snapshot into the intracellular bacterial composition of single-celled eukaryotes, which can be used to detect species-specific patterns and distinguish the bacterial groups that are enriched internally compared to the surrounding environment. This data can then be used to indicate and identify the presence of putative symbiotic interactions between eukaryotes and prokaryotes. It is necessary to take into account these species-specific patterns and interactions to have a comprehensive view on benthic nutrient cycling. For example, if foraminifera do harbour sulphur-cycle related endobionts, it may have implications for the benthic sulphur cycle as a whole, as foraminifera are in many areas the key components of the benthic eukaryote biomass.

In addition to gaining new insights on benthic ecology at species-level, metabarcoding methods can be applied into studying whole communities and changes within them.

This is particularly important from the biomonitoring-perspective, and the pioneering metabarcoding studies have already paved the way for the application of this method not only in research but also in day-to-day environmental conservation work and monitoring. The immense progress in the applicability and cost-effectiveness of NGS approaches combined with the increasing economic pressure on research budgets, the lack of skilled taxonomic expertise and the growing need for fast-generated large data sets makes metabarcoding a compelling option. Efforts to establish DNA metabarcoding in official programmes are currently taking place, and most likely in the coming decade we will see increasingly more DNA-based monitoring initiatives globally (European Marine Board, 2019). Monitoring can include, for example, tracking temporal and spatial changes in whole bacterial or eukaryote communities, or focusing on a specific indicator species. The biggest challenge left to tackle is standardization of the methods and a lack of uniform, coherent practices (McGee et al., 2019, Zinger et al., 2019). Currently, there exist numerous methods for DNA extraction, amplification, and analysis of sequence data (McGee et al., 2019). Data generated and analysed in different ways may be able to target the same prevailing trends but for monitoring purposies, a standard operation protocol is required for the sampling methods for bioinformatic analysis. In the case of biomonitoring water column eukaryote communities, optimized protocols and attempts at standardization have already emerged (Jeunen et al., 2019) but for the benthic environment, this is yet to be determined.

Molecular ecology methods, such as DNA metabarcoding, are slowly reforming the field of marine benthic research. This development is accelerated by constantly improving technologies and decreasing technical costs, making these methods more and more accessible. Some challenges certainly remain in streamlining and standardizing these approaches, and it is important to keep in mind potential pitfalls and biases. However, the potential of metabarcoding applications in benthic research is still underutilized, and it holds many exciting possibilities. Perhaps adding this piece to the puzzle may be the key in gaining a more comprehensive view on the whole benthic ecosystem, allowing us to discover new organisms and interactions.

6. References

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